Comm.
Dr. Loosli
Dr. Sommers
Dr. Wyatt

CHRONIC PULMONARY DISEASES

#906

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022

APR 10 1973

Application For Research Grant

Date: March 28, 1973

1. Name of Investigator(s): (include Title and Degrees)
Robert E. Brooks, Ph.D., Associate Professor of Pathology
Richard D. Moore, M. D., Professor and Chairman, Department of Pathology

2. Institution & Address:
University of Oregon Medical School
3181 S. W. Sam Jackson Park Road
Portland, Oregon 97201

3. Short Title of Project:

Morphological Studies on Induced Pulmonary Emphysema in Rabbits

4. Proposed Starting Date: July 1, 1973

5. Anticipated Duration of this Specific Study: Three years

6. Brief Descripton of Objectives or Specific Aims:

Spontaneous emphysema in old rabbits has been reported by two groups of investigators. Emphysema also occurs in rabbits as a sequela produced by intravenous injection of Freund's adjuvant (Moore, R. D., unpublished observations).

The objectives of the proposed study are: 1) the careful, morphological documentation of changes that occur in rabbit lungs following intravenous injection of complete Freund's adjuvant; 2) morphological examination of lungs from rabbits which inhaled whole cigarette smoke and gas-vapor phase from cigarette smoke before, during, or after development of pulmonary emphysema due to intravenous injection of complete Freund's adjuvant; and 3) morphological comparison of lungs from the above two experimental groups with lungs of rabbits treated either with compounds that increase or decrease the lung macrophage response to circulating complete Freund's adjuvant or with compounds which produce primary injury to the alveolar capillary circulation.

On the basis of what is learned from these studies, we expect to be able to determine if cigarette smoke (whole or gas-vapor phase) affects the onset, development or course of adjuvant-induced emphysema in the rabbit. And, if it appears that cigarette smoke does affect this animal model, whether the effect is likely to be due to action on alveolar macrophages, action on the pulmonary microcirculation, or on both.

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7. Give a Brief Statement of your Working Hypothesis: We hypothesize that adjuvant-induced emphysema of the rabbit will provide a profitable animal model for the study of the relation of cigarette smoke inhalation to pulmonary emphysema.

8. *Details of Experimental Design and Procedures: (Attach Separate Pages)*

Appended.

9. Physical Facilities Available (Where Other than Administering Organization Indicate Geographical Location)

Appended.

10. Additional Requirements:

A suitable method for providing cigarette smoke inhalation to rabbits will have to be determined. We will need to purchase, for the second year of this project, a Walton Horizontal Smoke Exposure Machine (or equivalent). We have been in correspondence with Dr. John H. Kreiser, of the Council, regarding the Walton smoke machine. During the first year of the project, we will need to obtain advice from various sources and possibly carry out pilot studies on rabbits in order to work out procedural problems.

11. Biographical sketches of all principal and professional personnel (append)

Appended.

12. List of publications: (Five most recent as pertinent) (append)

Appended.

13. Budget: (1st year)

A. Salaries (Personnel by names)	% time	Amount
Professional		
Robert E. Brooks, Ph.D.	15%	---
Richard D. Moore, M. D.	10%	---
		REDACTED
Technical		
Research Assistant (to be hired)	100%	
*@ $600/mo + 13% payroll assessment ($7200 + $936)		
	Sub-Total	REDACTED
B. Consumable Supplies (list by categories)		
Laboratory chemicals: tissue fixatives, tissue embedding materials, stains, alcohols		500
Microtomy supplies: glass for microtome knives, specimen grids		200
Photographic supplies: plates, developers, fixers, papers		700
	Sub-Total	1,400
C. Other Expenses (itemize)		
Rabbits, purchase, about 70 animals @ $5.00/animal		350
Animal care for rabbits for entire first year		2,300
1 only diamond microtome knife, 3.00 mm size		560
	Sub-Total	3,210
D. Permanent Equipment (itemize)		
None		
E. Overhead (15% of A+B+C)		1,912
	Total	14,658

Estimated Future Requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Overhead	Total
Year 2	REDACTED	$1,500	$2,500	$2,500	$1,881	$16,924
Year 3		$1,500	$2,500	---	$1,943	$14,893

It is understood that the applicant and institutional officers in applying for a grant have read and found acceptable the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Signature Robert E. Brooks
Director of Project
Telephone REDACTED

Signature
Business Officer of the Institution
Telephone

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Other Sources of Financial Support

List financial support for research from all sources, including own institution, for this and/or related research projects.

Current

Title of Project	Source	Amount	Duration
Electron Microscopic Study of Lung Alveolar Surface	Medical Research Foundation of Oregon	$2,000	3-1-72 through 5-31-73

Pending

Title of Project	Source	Amount	Duration
Development of an Electron Microscopy Program to Aid in Cancer Diagnosis	Milheim Foundation for Cancer Research	$9,669	7-1-73 through 6-30-74

8. EXPERIMENTAL DESIGN AND PROCEDURES

The first year of the proposed research will be primarily concerned with establishing a baseline of development of adjuvant-induced emphysema in the rabbit.

Rabbits will be prepared by the injection of 1 ml of complete Freund's adjuvant (CFA) by way of the marginal ear vein. For this study 5- to 6-month-old rabbits of both sexes will be used. Animals will be killed, by Nembutal injection, at weekly intervals up to 12 weeks following injection of CFA. Three animals will be used for each time point. After 12 weeks, rabbits will be sacrificed at three week intervals, up to 24 weeks. Untreated rabbits will be sacrificed at 6, 12, 18 and 24 week intervals. Six additional experimental and four additional control animals will be carried along to replace any rabbits that die during the experimental period of 24 weeks. Therefore, a total of 70 animals will be used during the first year of the project.

When the chest of each animal is opened at time of sacrifice, the great vessels of the heart will be clamped, the trachea exposed and cut, and the lungs allowed to collapse. A glutaraldehyde fixative solution will be introduced into the lungs through the trachea and the lungs allowed to expand with fixative until they fill the chest cavity. The trachea is tied off and the lungs removed from the chest and immersed in a container containing the same fixative. Two to 4 hours later, small pieces of peripheral lung are removed and postfixed with osmium tetroxide solution for electron microscopy. The following day, slices of remaining lung are cut about 2 mm thick with a special double-bladed knife or by meat slicer and the resultant slices examined on a lighted box by dissecting microscope for "subgross" morphology. Some of these slices and other thicker pieces are also prepared in the usual way for subsequent light microscopic examination.

Lung tissue from each rabbit will be studied at the gross, subgross, light and electron microscopic levels. Photographic and descriptive records will be made and analyzed in terms of tissue reactions.

Earlier studies have shown that lung tissue reaction to CFA consists mainly of exudation of macrophages into the air spaces and granuloma formation within the tissue of the lungs. Subsequent resolution of the exudate and granulomas leads to alveolar wall destruction. The ultra-structural events related to this alveolar wall destruction have not been followed closely. One of the main purposes of this proposed project is the detailed description of fine structural changes occurring in the lungs of the CFA-treated rabbits.

Information obtained from the first year studies will be used as a basis for conducting the second and third year investigations.

We anticipate that some optimal time will be found when all CFA-treated rabbits will show definite but not excessive alveolar wall destruction. This time, possibly 12 weeks post CFA injection will be used as a point about which the smoking experiments will be run. For example, taking the

12 weeks point, a group of 3 animals will receive whole cigarette smoke or gas-vapor phase from time zero (when CFA is injected) to 6 weeks post injection. A second group of 3 animals will be treated in the same way, except that they will inhale whole cigarette smoke or gas-vapor phase from the 9th through 15th weeks. A third group will be exposed from the 15th through the 21st weeks. "Control" rabbits, untreated with CFA, will be exposed to cigarette smoke in parallel, but not necessarily at the same time, as the CFA-treated rabbits. The number of weekly smoke exposures and duration of each exposure will have to be determined by pilot studies. Rabbits will be sacrificed at 24 weeks from the start of the experiment. The lungs will be handled as in the first year studies. Findings will be compared to the lungs of the first year project.

Third year studies are based on reports that whole cigarette smoke and gas-vapor phase influences the alveolar macrophages (1) and the lung microcirculation (2) respectively.

In order to determine the importance of the macrophage, and particularly its enzyme content, in the development of emphysema, compounds that delay or speed up the release of its cytoplasmic contents will be used. Their influence will be determined by modifications of the rate of development and severity of emphysema.

The animals will be given CFA as before. Beginning at approximately 7 days, when the macrophage response to the adjuvant is well developed, animals will be treated with stabilizers and labilizers and with stimulators and depressors of macrophage activity. Vitamin A (3, 4), carageenan (5) and streptolysin S (6) will be used as labilizers; chloroquine (7, 8) and hydrocortisone (9, 10) as stabilizers; glucan as a stimulator; and, methyl palmitate as a depressor (11). Appropriate controls will be used. Most of these agents, particularly in large quantities, are associated with multisystem side effects. The experiments will, of necessity, be controlled by paired animals--one receiving adjuvant plus added compound and the other only added compound. Gross and optical microscopic survey of the principal organ systems will be mandatory in these paired animal experiments.

Even if the accumulation of leukocytes and/or macrophages is important in the development of emphysema, their appearance is dependent on prior alterations of the microcirculation. There must be change in permeability, slowing of blood flow, margination and migration of white cells, platelet and local oxygen deficit. Therefore, alteration of the microcirculation should be investigated as an underlying cause of emphysema.

Incomplete Freund's adjuvant plus a capillary-damaging compound such as sodium tetradecyl sulfate will be used as a means of injuring the pulmonary microcirculation. The oil emulsion emboli reach this level of the circulation, persist for a short period, and should allow action by the capillary-damaging agent. Pilot studies will be run on a small

number of animals using intravenous injection of adjuvant plus one of several trial levels of sodium tetradecyl sulfate. We are uncertain of the results of such an approach until tested and hesitate to generate a protocol beyond that listed for the study of emphysema above.

References:

1. Pratt, S. A., Smith, M. H., Ladman, A. J., and Finley, T. N. The ultrastructure of alveolar macrophages from human cigarette smokers and nonsmokers. Lab. Invest. 24: 331, 1971.

2. Williams, J. B., and Anderson, W. H. Acute effects of cigarette smoke on pulmonary ventilation-perfusion relationship. Amer. Rev. Resp. Dis. 98: 145, 1968.

3. Weissman, G. Labilization and stabilization of lysosomes. Fed. Proc. 23: 1038, 1964.

4. Shamberger, R. J. Inhibitory effect of vitamin A on carcinogenesis. J. Nat. Cancer Inst. 47: 667, 1971.

5. Cantanzaro, P. J., Schwartz, H. J., and Graham, R. C. Spectrum and possible mechanism of carrageenan cytotoxicity. Amer. J. Path. 64: 387, 1971.

6. Weissman, G., Becker, B., Wiedermann, G., and Bernheimer, A. W. Studies on lysosomes. VII. Acute and chronic arthritis produced by intra-articular injections of streptolysis S in rabbits. Amer. J. Path. 46: 129, 1965.

7. Abraham, R., and Hendy, R. Effects of chronic chloroquine treatment on lysosomes of rat liver cells. Exp. Molec. Path. 12: 148, 1970.

8. Read, W. K., and Bay, W. W. Basic cellular lesion in chloroquine toxicity. Lab. Invest. 24: 246, 1971.

9. Deodhar, S. D., and Bhaqwat, A. G. Desquamative interstitial pneumonia-like syndrome in rabbits. Arch. Path. 84: 54, 1967.

10. Merkow, L., Pardo, M., Epstein, S. M., Verney, E., and Sidransky, H. Lysosomal stability during phagocytosis of Aspergillus flavus spores by alveolar macrophages of cortisone treated mice. Science 160: 79, 1968.

11. Lentz, P. E., and DeLuzio, N. R. Biochemical characterization of Kupffer and parenchymal cells isolated from rat liver. Exp. Cell Res. 67: 17, 1971.

Illustrations given in Appendix, page 11.

9. PHYSICAL FACILITIES AVAILABLE

General facilities available are those of the Department of Pathology and the School of Medicine of the University of Oregon.

A fully equipped electron microscope laboratory is in the Department of Pathology. This laboratory has an RCA EMU-3G and a Philips EM 200 electron microscope. Each electron microscope is housed separately and each has its own attached darkroom for developing negatives. The laboratory also contains a darkroom with all necessary equipment for enlarging the negatives to make final prints. In addition, there are present four automatic microtomes for producing sections for electron microscopy, a mechanical glass knifemaker, a vacuum evaporator, and a Smith-Farquhar tissue chopper.

General purpose laboratories are available in new departmental facilities.

Housing for small animals is adjacent to the pathology laboratories and a veterinarian is in attendance in the animal care facility.

11. BIOGRAPHICAL SKETCHES

a. Name; Title; Birthdate:

Robert E. Brooks, Ph.D.
Associate Professor of Pathology
REDACTED

b. Role in project:

Principal investigator.

c. Education; honors:

University of California, Los Angeles REDACTED (physics)
University of Oregon Medical School, athology)
University of Oregon Medical School, pathology)
Recipient of a Special Fellowship from the National Cancer Institute, 1965-66.

d. Professional experience:

1972-present Associate Professor of Pathology, University of Oregon Medical School.
1967-1972 Assistant Professor of Pathology, University of Oregon Medical School.

1961-1967 Instructor (electron microscopy), University of Oregon Medical School.
1950-1961 Laboratory Technician, Department of Pathology, University of California Medical School, San Francisco.
1948-1950 Laboratory Technician, Atomic Energy Section, University of California at Los Angeles.

a. Name; Title; Birthdate:

Richard D. Moore, M. D.
Professor and Chairman, Department of Pathology
REDACTED

b. Role in project:

Co-investigator.

c. Education; honors:

Gonzaga University, Spokane, Washington.
Western Reserve University Medical School, Cleveland, Ohio, M. D., 1947.
Sigma Xi

d. Professional experience:

1969-present Professor and Chairman, Department of Pathology, University of Oregon Medical School.
1967-1969 Professor of Pathology, Case Western Reserve University, Cleveland, Ohio.
1961-1969 Associate Pathologist, University Hospitals, Cleveland, Ohio.
1957-1967 Associate Professor of Pathology, Western Reserve University.
1956-1957 Assistant Professor of Pathology, University of Rochester, Rochester, New York.
1955-1956 Assistant Professor of Pathology, Western Reserve University.
1954-1956 Assistant Pathologist, University Hospitals, Cleveland.
1953-1955 Senior Instructor in Pathology, Western Reserve University.
1952-1953 Instructor in Pathology, Western Reserve University.

12. LIST OF PUBLICATIONS

a. Robert E. Brooks:

Brooks, R. E. Lung alveolar cell cytosomes: A consideration of their significance. Zeit. Zellforsch. 106: 484-497, 1970.

Brooks, R. E. Ultrastructural evidence for a noncellular lining layer of lung alveoli: A critical review. Arch. Intern. Med. 127: 426-428, 1971.

Brooks, R. E., and Addison, B. Intracellular components of neoplastic and normal alveolar cells from mouse lungs: Quantitative ultrastructural comparison. J. Nat. Cancer Inst. 47: 639-644, 1971.

Brooks, R. E. Lung surfactant: An alternate hypothesis. Amer. Rev. Resp. Dis. 104: 585-586, 1971.

Brooks, R. E. Ultrastructure of lung lesions produced by ingested chemicals. I. Effect of the herbicide paraquat on mouse lung. Lab. Invest. 25: 536-545, 1971.

b. Richard D. Moore:

Moore, R. D., Lamm, M. E., Lockman, L., and Schoenberg, M. D. Cellular aspects of the action of Freund's adjuvant in the spleen and lymph nodes. Brit. J. Exp. Path. 44: 300-311, 1963.

Moore, R. D., and Schoenberg, M. D. Alveolar lining cells and pulmonary reticuloendothelial system of the rabbit. Amer. J. Path. 45: 991, 1006, 1964.

Moore, R. D., and Schoenberg, M. D. The response of the histiocytes and macrophages in the lungs of rabbits injected with Freund's adjuvant. Brit. J. Exp. Path. 45: 488-497, 1964.

Schoenberg, M. D., Stavitsky, A. B., Moore, R. D., and Freeman, M. J. Cellular sites of synthesis of rabbit immunoglobulins during primary response to diphtheria toxoid-Freund's adjuvant. J. Exp. Med. 121: 577-590, 1965.

Moore, R. D., and Schoenberg, M. D. A comparison of the primary and secondary response to complete Freund's adjuvant. Brit. J. Exp. Path. 47: 60-69, 1966.

APPENDIX

Illustrations

The figures are light micrographs taken of histological sections from uninflated rabbit lungs. Treated animals received a single intravenous injection, by ear vein, of 1.0 ml complete Freund's adjuvant. All micrographs are the same magnification, 72 X.

Figure Legends

Fig. 1. Untreated animal.

Fig. 2. Two weeks after receiving adjuvant there are numerous collections of macrophages within air spaces and histiocytes in the walls. Distinct granulomas are present with a central collection of epithelioid cells and collar of lymphocytes.

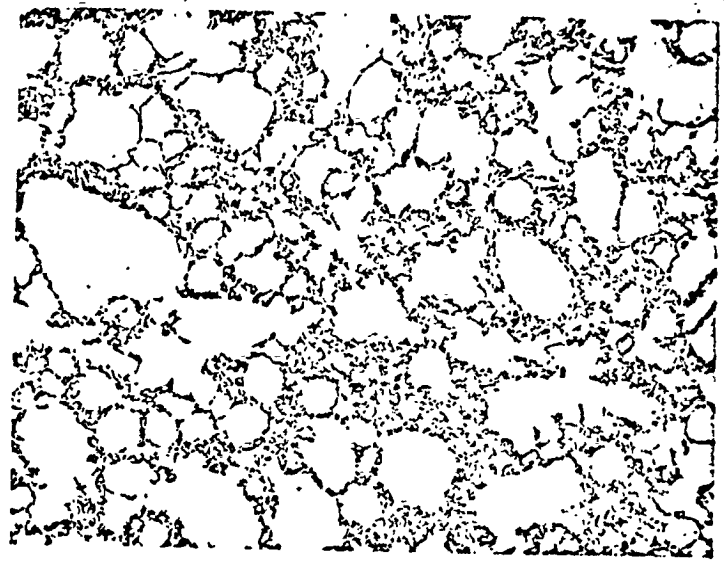
Fig. 3. Four weeks after receiving adjuvant, the inflammatory changes are similar to those seen at two weeks. The granulomas dominate the inflammatory reaction at this time.

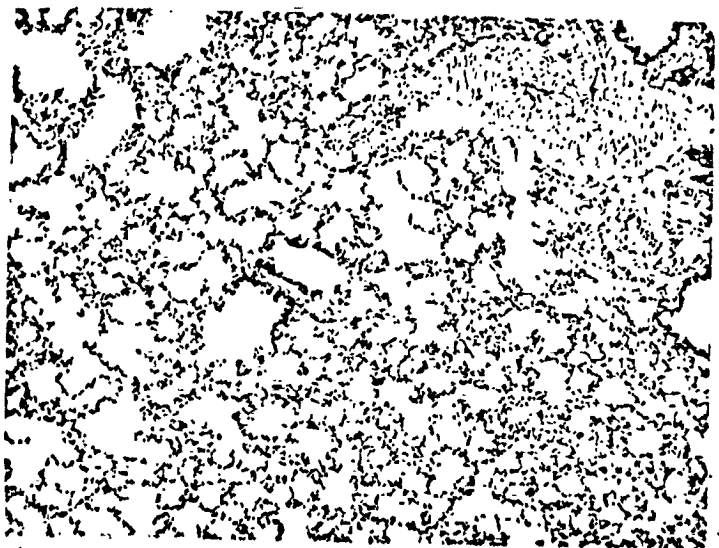
Fig. 4. Eight weeks after receiving adjuvant much of the exudate has been cleared from the lung. Many of the terminal air spaces are distended. This is more apparent when compared with Fig. 1.

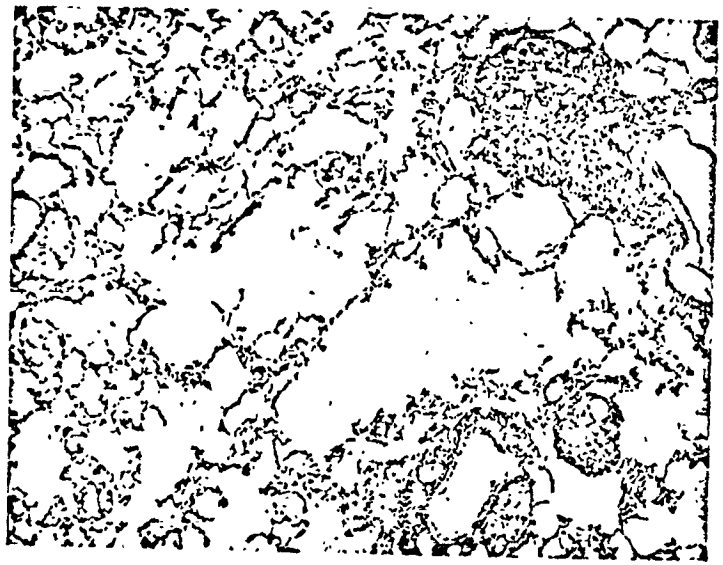
Fig. 5. Untreated animal.

Fig. 6. This photomicrograph is also from an animal eight weeks after adjuvant. It is similar to Fig. 4, but illustrates two residual granulomas.

Figs. 7 & 8. These illustrations are from animals twelve weeks after receiving adjuvant. There is still some residual inflammatory exudate in Fig. 7 and a small granuloma in Fig. 8. The distention of the terminal air spaces persists. Compare with Fig. 5 which is an illustration of the lung from an untreated animal.







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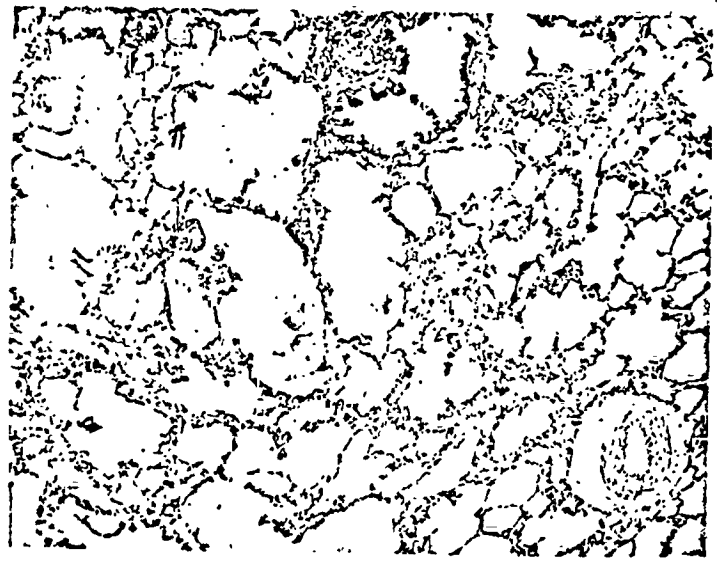

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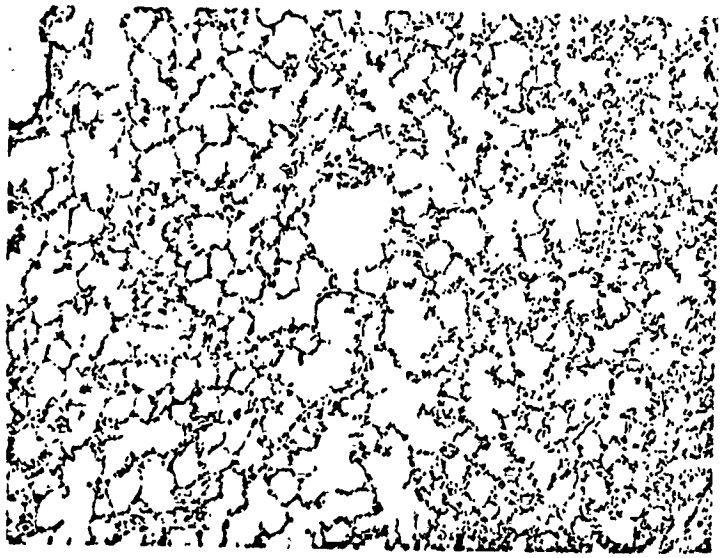

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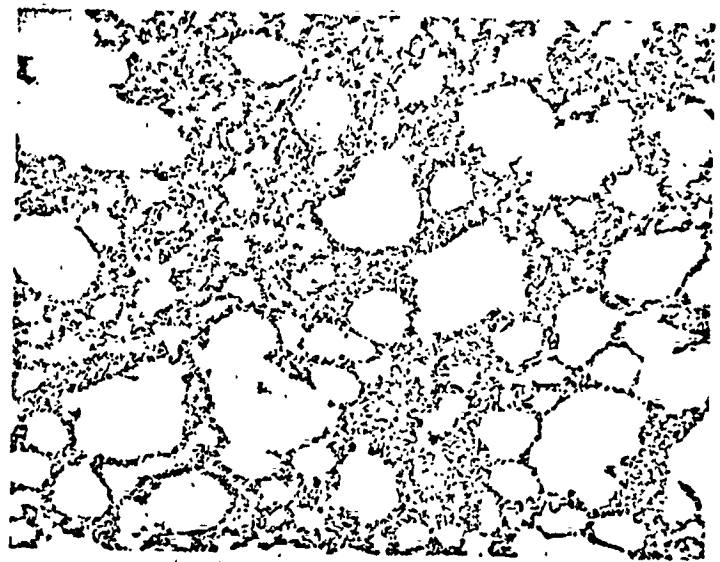

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